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COMPLETE SPECIFICATION

Urinary Estrogen Compositions and methods for preparing them

We, AYERST, MCKENNA & HARRISON, LIMITED, a corporation organized and existing under the laws of the Dominion of Canada, of Montee St. Laurent, Province of Quebec, Dominion of Canada, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to the preparation of hormone compositions and, more particularly, to the preparation of estrogenic compositions of substantial purity and low toxicity.

An object of the invention is to provide a process for obtaining an estrogenic product of high potency and relatively low toxicity. Another object is to prepare compositions from equine urinary material of such purity as to be capable of use as an intravenous pharmaceutical by means of said process. It is a further object of the invention to prepare an estrogenic product from equine urine by the hereinafter described process which is substantially free of urinary odors and capable of use as a topical medicinal. A further object is the preparation of an estrogenic hormonal product by the inventive process which is orally effective for the treatment of the menopausal syndrome.

Processes are known which produce water-soluble estrogenic compositions from pregnant mares' urine. However, none of these known processes is capable of producing a highly purified estrogenic product that is free of potentially toxic impurities or such a product that is substantially non-odorous. Moreover, no procedure has previously been described for producing an estrogenic product from pregnant mares' urine that is of such purity and estrogenic content as to be capable of use as an intravenous medicinal.

In addition to the conjugated estrogens present in pregnant mares' urine, it should be realized that there are many natural substances in the urine that have been rendered physiologically non-toxic but these same substances become highly toxic when complexes are broken down, or certain substances such as the phenolic compounds are reduced to free bases or acids instead of salts, either through bacterial action or by the treatment which seeks to isolate the estrogenic potency.

It is obvious that a parenteral preparation must be non-toxic at the dosage levels used and this is particularly important when considering an intravenous preparation. To obtain an intravenous medicinal from equine urine containing not only high amounts of foreign proteins but pyrogens, various amines and diamines, indoxyl, or free phenolic and cresylic compounds is a difficult problem; and the problem is often further complicated by the presence of substantial bacterial contamination in the raw urinary fluids at the time of its collection.

The known procedures for extracting conjugated estrogens from pregnant mares' urine, while producing products of satisfactory potency for certain purposes, were not capable of yielding products that could be used intravenously without further treatments. The present invention deals with a new method for obtaining special preparations requiring high estrogenic concentration and low toxicity. As will be described, the present invention discloses an improved method for preparing intravenous compositions as well as other preparations requiring the use of high potency, substantially unhydrolyzed water-soluble estrogenic conjugates practically free from all toxic elements that prohibit its use for the purposes intended.

In carrying out the improved method for obtaining the estrogenic product of the invention, it is first necessary to start with an estrogenic concentrate derived from pregnant

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mares' urine. This initial concentrate may be obtained by known procedures. Following the preliminary concentration and purification operations, the concentrate, dissolved in an aqueous medium, is then subjected to a salting-out step wherein the estrogens are precipitated in relatively pure form, leaving a substantial amount of impurities dissolved in the liquid medium.

The earliest procedure for initially concentrating the estrogens from pregnant mares' urine involved selective extraction of the estrogens using activated carbon, fuller's earth or other suitable adsorbing agent. The estrogens were selectively adsorbed and these were removed from the agent by extraction with an organic solvent, such as pyridine, aniline, morpholine and various organic amines. Preferably the adsorbing agent was charcoal and the solvent was pyridine. Following the solvent extraction, the extract was then concentrated by heat and preferably purified.

A better method for initially concentrating the estrogens in pregnant mares' urine involves the use of an organic solvent for directly extracting the estrogenic elements in unhydrolyzed pregnant mares' urine. Thus butanol may be used as the extracting agent. However, a more preferred solvent extraction procedure for initial concentration involves the extraction of the water-soluble estrogenic orally active substances from substantially unhydrolyzed equine urine utilizing an organic solvent of the type that is substantially water-insoluble. The solvent used is an alcohol or ketone having a water-solubility of less than about 6% and a preferred solvent is hexanol although other solvents such as cyclohexanol, methyl-cyclohexanol, cyclohexanone or isophonone could be used. The estrogenic materials are selectively taken up by the solvent leaving behind substantially all of the proteinaceous material and large amounts of the high molecular weight aliphatic amines and diamines. By this procedure, a product may be obtained containing about 20%, or somewhat more, estrogenic components but still containing potentially toxic substances.

Alternatively, instead of using alcohols or ketones of limited water solubility as described above, one may selectively remove the estrogenic potency from equine urinary material by first reacting the estrogens to form amine salts and then extracting with benzene or with a chlorinated aliphatic solvent. This concentration procedure involves the reaction of the estrogens in the equine urinary material with a nitrogenous base salt, thus forming estrogenic salts of alkyl amines, unsubstituted cycloalkyl amines, unsubstituted mono-cyclo aromatic-hydrocarbon amines, p-amino-acetophenone, dimethyl aminotoluene, procaine, pyridine, aminopyridines and mixtures thereof. After forming the amine salts of the conjugated estrogens, they are thereby rendered soluble in an organic solvent such as benzene or low molecular weight liquid chlorinated aliphatic hydrocarbons, and one of these solvents selectively separates the estrogens from the undesired urinary matter. Among the solvents mentioned, ethylene dichloride is preferred.

Following any of the concentration procedures described above, the estrogenic factors are removed from the solvent and may be treated to remove inorganic salts, odoriferous phenols and other undesired urinary substances by use of further selective solvent extractions. In general, however, the product from any of the concentration procedures mentioned hereinabove still has such impurities present as to make the product at this point potentially dangerous for intravenous use. The product still contains pyrogens, indoxyl, and cresol compounds. It has been found that these substances should be removed in order to obtain a product of sufficient purity and low toxicity as to permit intravenous administration without dangerous side-effects.

The partially purified product obtained after carrying out the extractive concentrations referred to above is now substantially improved by treatment with an aqueous saturated salt solution utilizing an alkali metal sulfate, phosphate, or chloride, the sodium and potassium salts being preferred. This step has been found to selectively precipitate the estrogenic potency leaving in the aqueous solution substantially all of the toxic materials herebefore mentioned. The precipitate is filtered and is extracted with an aliphatic ketone containing a small amount of an alcohol which selectively dissolves the estrogenic conjugates to the exclusion of the major amount of the inorganic salt used for precipitation. As a final step, water is added to the solvent solution and the product is concentrated to remove substantially all of the solvent leaving the concentrate in aqueous solution. This new and more highly concentrated product may either be utilized in the aqueous state in the preparation of the intravenous product or directly incorporated while in aqueous solution in an ointment base or it may be dried under vacuum conditions, preferably by lyophilization (freeze-drying), prior to utilization for the particular purpose desired.

In treating the partially purified estrogenic concentrate with the salt solution and in all of the succeeding steps, it is necessary to exclude as well as remove pyrogens where an intravenous product is to be made. Stainless steel equipment and all glassware must be made absolutely clean to prevent contamination with pyrogens and double distilled water must be used in all aqueous solutions.

Besides the removal of indoxyl and cresylic compounds, as well as pyrogens, an important factor involved in the preparation of an intravenous product from equine urine is that

of stability. In relatively crude extracts, natural stability factors are apparently present since it has been found that these products have a relatively high order of stability as compared to more highly purified estrogenic compositions. In the preparation of an intravenous product utilizing the novel, high potency estrogenic product, it has been discovered that a requirement of such composition is for an agent to maintain the stability of the conjugates and prevent their destruction. Relatively pure estrogenic conjugates are highly unstable particularly in the presence of moisture. Where moisture is present, or under aqueous conditions, an acid environment forms which quickly destroys the product. An important discovery is the finding that buffering agents, capable of maintaining a substantially neutral environment with pH from 6.5 to 7.5, prevent deterioration of the estrogenic composition.

Buffering or stabilizing agents useful with the estrogenic conjugates of the invention are the alkali metal salts of organic or inorganic acids which are capable of maintaining a substantially neutral pH in the range of 6.5 to 7.5 with a preference to the slightly alkaline side. Any of the well-known buffering salts or buffering mixtures may be utilized provided they are substantially non-toxic in the amounts used and are capable of maintaining the desired hydrogen ion concentration.

As examples of preferred buffering mixtures may be mentioned sodium or potassium dihydrogen phosphate together with either sodium or potassium hydroxide, disodium or dipotassium mono-hydrogen phosphate or trisodium or tripotassium citrate. Another buffer mixture suitable for use in the present invention is either sodium or potassium monohydrogen phosphate together with hydrochloric acid, sulfuric acid, citric acid or disodium or dipotassium citrate. Another buffer mixture may be disodium or dipotassium citrate together with trisodium or tripotassium citrate, or in lieu of the latter, sodium or potassium hydroxide. One may also use trisodium or tripotassium citrate with either citric acid, hydrochloric acid, or sulfuric acid. As another example, potassium hydrogen phthalate with sodium or potassium hydroxide or dipotassium phthalate with hydrochloric acid or sulfuric acid are also effective. Many other buffering materials as taught in the text "Determination of Hydrogen Ions" by W. M. Clark may be used besides those mentioned herein provided they are substantially non-toxic and effect a hydrogen ion concentration in the pH range indicated.

For intravenous preparations, the minimal concentration of stabilizing or buffering agent necessary to attain good stability is $^1/_{100}$ molar. Considerably higher amounts may be used without deleterious effect up to the point of causing salting out. A preferred range, however is between $^1/_{10}$th and $^1/_{20}$th molar. The molar concentration, of course, refers to either a single buffering material or a combination or mixture of buffering materials.

In order to permit the drying of the estrogenic concentrate which is quite hygroscopic, and, in addition, to obtain a desirable bulk for intravenous injection, it is also necessary to provide a carrier which is soluble and, of course, non-toxic in blood fluids. Such a carrier and drying aid is preferably lactose although one may also use dextrose, galactose, pectin, gelatin, dextran, polyvinylpyrrolidone, or any other agent capable of acting as a carrier for the estrogenic potency, permitting easy drying of the product, and having the characteristics noted above.

The intravenous composition has been found especially useful as a hemostatic agent as, for example, in the treatment of functional uterine bleeding or to control excessive bleeding generally. In the case of functional uterine bleeding, the usual treatment consists of either curettage, where rapid hemostasis is required, or the oral ingestion of estrogenic conjugates, where an emergency condition is not present. In the latter case, control of bleeding is effective within two to five days.

By the use of the estrogenic product of the invention, the intravenous preparation is substantially more effective since hemostasis will take place within two to eight hours.

The intravenous preparation is also deemed of value in the treatment of atherosclerosis. It has been found that as little as about 0.1 of a milligram per kilogram of body weight, administered intravenously once a week, will restore cholesterol metabolism to normal, and restore disturbed ratios of phospholipids to cholesterol, and ratios of alpha-lipo-protein to beta-lipo-protein. For such disease, synthetic estrogens are ineffective while oral or parenteral estrogens require such high levels as to result in such untoward side effects as to prohibit their use.

Still another use for the highly purified estrogenic product is its utilization, suitably buffered, in an ointment or cold cream base carrier for the topical treatment of *Acne vulgaris*. Because of the freedom from a large proportion of objectional urinary solids but particularly because of the freedom from low molecular weight phenols and cresols, the estrogenic product may be incorporated in a cream for topical use whereas earlier products of this type were so odoriferous as to limit their use as a topical medicament.

In addition to the utilities noted above, the estrogenic product of the invention may be utilized as an oral medicament in the form of tablets for the treatment of the menopausal syndrome. In preparing the tablets, the estrogenic product is combined with a buffering agent and sufficient carrier is incorporated to provide a tablet of suitable size and potency.

Tablets containing from 0.625 to 2.5 milligrams of the conjugated estrogens (expressed as sodium estrone sulfate), when taken daily, have been found effective in practically every case to give substantial relief from the common, autonomic, emotional and mental disorders of the menopausal syndrome. Tablets utilizing the product of the invention cause fewer side reactions, such as nausea, than prior substances.

In preparing the intravenous product, one combines the estrogenic concentrate of the invention with the buffering material and water-soluble carrier, all dissolved in water. The pH of the solution is carefully checked and adjusted to within a pH of 6.5 to 7.5 with a preferred pH from 7.2 to 7.4. The solution is filtered through a bacteriological filter, placed in vials and dried by lyophilization. This product, when reconstituted with sterile pyrogen-free water, is suitable for intravenous injection.

In preparing the topical medicaments, the purified estrogenic concentrate is combined in a blender or mixer with an ointment base or vanishing or cold cream base carrier. A typical ointment base material comprises glyceryl monostearate, cetyl alcohol, glycerine, spermaceti, methyl stearate, methyl-p-hydroxy benzoate and p-chloro-meta-resol. In the standard non-drying cold cream bases, a typical mixture is glycerol-titanium dioxide suspension, hydrated magnesium aluminum silicate, glyceryl monostearate, cetyl alcohol, beeswax, spermaceti, methyl stearate, heavy mineral oil, propylene glycol monostearate, sodium lauryl sulfate, sodium benzoate and methyl-p-hydroxy benzoate.

Another form of the topical medicament is the liquid type containing alcohol, glycerol and a buffering agent to secure a pH substantially in the range of neutrality and preferably on the slightly alkaline side.

The following examples illustrate the invention in greater detail. It should be clearly understood that these examples are essentially for illustrative purposes and are not intended to be limitative of the invention.

EXAMPLE 1.

100 gallons of pregnant mares' urine, suitably preserved to prevent material hydrolysis, is extracted with 40 gallons of hexanol. The spent urine is removed and the hexanol extract is washed with 4 gallons of 8% sodium hydroxide in water. The aqueous phase is discarded. The washed hexanol is concentrated *in vacuo* to 2 gallons. To the hexanol concentrate is now added 2 volumes (approximately 4 gallons) of hexane. The alcohol-hydrocarbon mixture is then washed once with ½ gallon and twice with ¼ gallon of water. The estrogenic potency goes into the water solution and the hexane-hexanol fraction is discarded. The crude aqueous concentrate is adjusted to a pH between 7.0 and 7.5, diatomaceous earth is added and the mixture is dried *in vacuo*.

The dried cake is dissolved in a minimal amount of methanol which is then concentrated *in vacuo* to approximately 6 to 10 liters. The methanol solution is then poured into 15 volumes of acetone (15 times the volume of methanol solution). The precipitate which forms is filtered and washed with 97% acetone and 3% methanol, the washed liquid being added to the filtrate. The filtrate is now concentrated *in vacuo* to a small volume, 10 liters of water are added and the concentration is continued to remove the organic solvents.

The concentrate is made up to 10 to 12 liters of water. The pH of the solution is adjusted to approximately 5.0 with hydrochloric acid and the acid solution is rapidly washed with one wash of ¼ volume benzene and another wash of ¼ volume of ether. The wash liquids are discarded. Water is added, the solution is neutralized and adjusted to approximately pH 7.5 with sodium hydroxide solution, and the product is distilled to remove organic solvents and to reduce the product to approximately 10 to 12 liters of aqueous, partially purified concentrate.

To the aqueous concentrate is now added 2 volumes (20 to 24 liters) of saturated sodium chloride solution which precipitates the potency. A filter aid such as diatomaceous earth is added and the estrogenic precipitate is separated from the aqueous medium by filtration. This precipitate is extracted with 97% acetone and 3% methanol. Water is added to the organic solvent solution and the mixture is concentrated *in vacuo* to give approximately 2.5 gallons of water free of organic solvents and containing the conjugated estrogens in purified form. The pH of the solution is adjusted to approximately 7.0 to 7.5.

EXAMPLE 2.

About 100 gallons of suitably preserved pregnant mares' urine is extracted with 40 liters of ethylene dichloride containing 1% dicyclohexylamine acetate by weight. The solvent extract is washed three times with 1 gallon and once with a half gallon of 2% sodium hydroxide solution in water. The aqueous alkaline extract is washed with a small amount of ethylene dichloride and the spent chlorinated solvent together with the wash liquid is discarded. The aqueous solution is adjusted to a pH of 7.5 and is concentrated *in vacuo* to dryness forming a dry cake which may then be treated in the manner disclosed in the preceding example.

If a higher purity of 90—95% or somewhat purer product is desired, the aqueous final concentrate as produced by the procedures described above is dried *in vacuo* and dissolved in benzene containing a minimal percentage of alcohol to effect solution. The solution is then passed through a column of activated alumina

which retains the estrogenic potency. Elution is carried out with the same solvent mixture as mentioned, preferably an aromatic hydrocarbon, such as benzene, combined with a lower aliphatic alcohol of 1 to 4 carbon atoms, the alcohol being present in the mixture to the extent of 10—50% by volume. The eluate is then concentrated *in vacuo*, the concentrate is taken up in water and is suitably buffered to a pH of about 7.5. While one may achieve about 95% purity or even higher by this procedure, a product of such purity is not of paramount importance for the uses described herein.

EXAMPLE 3.

To prepare an intravenous preparation, a purified liquid concentrate, prepared as described in the preceding Examples 1 and 2 and free of pyrogens, indoxyl, and cresol compounds, is combined with a buffering agent and a bulking agent or carrier.

About 6 to 8 liters of the estrogenic concentrate containing 15 to 20 milligrams per milliliter of conjugated estrogens (expressed as sodium estrone sulfate) is combined with 62 grams of sodium citrate dissolved in 4 liters of water to which has been added 250 grams of lactose. The aqueous solution is made up to 20 liters with double distilled water. The pH of the solution is adjusted to approximately 7.2 to 7.4 with 5% hydrochloric acid or sodium hydroxide as required. The buffered solution is filtered through a bacteriological filter, filled sterilely into vials and lyophilized. This product, when reconstituted with pyrogen-free double distilled water, is an effective intravenous medicament.

EXAMPLE 4.

To prepare a topical medicament for the treatment of *Acne vulgaris*, 2.5 liters of estrogenic conjugates containing 50 milligrams per milliliter (expressed as sodium estrone sulfate) is combined with 97 kilograms of standard ointment base to which has been added sufficient red coloring material to impart a flesh color to the product. Water is added in an amount to bring the entire product to 100 kilograms of cream. This is filled sterilely into jars or tubes and provides approximately 1.25 milligrams of conjugated estrogens per gram of ointment base.

EXAMPLE 5.

A topical liquid medicament also suitable for the treatment of *Acne vulgaris* is made up by combining the purified liquid estrogenic concentrate containing 93.75 grams of conjugates (expressed as sodium estrone sulfate) with 50 liters of glycerol and 2800 grams of sodium citrate. Sufficient 95% ethyl alcohol is added to give a final concentration of approximately 12.5% alcohol. Water is added to bring the product to 500 liters which is finally adjusted to a pH of approximately 7.3 to 7.5.

Many modifications may be made within the scope of the invention. Thus, other procedures for concentration of the estrogens from pregnant mares' urine may be carried out prior to the salting-out step as indicated earlier. Regardless of which concentration procedure is first utilized, the salting-out method for obtaining the estrogens as described hereinabove is a necessary and important element to obtain the highly purified and concentrated product. The product of the invention has been found to contain the majority of the female sex hormones in the form of sulfates. Identified compounds are the sulfates of estrone, equilin, equilenin, β-estradiol, α- and β-dihydroequilenin. These are combined with relatively non-toxic urinary solids, the estrogenic content being at least 40% by weight, and, as described in the above examples, ranging from 40% to 50% by weight, and comprising the sulfates of estrone, equilin, α- and β-dihydroequilenin, equilenin, and β-estradiol.

The acetone-methyl alcohol solvent utilized after the salt precipitation step is essentially to separate the estrogenic potency from the inorganic salts. In place of acetone, one may use any lower aliphatic ketone such as methyl-ethyl ketone. Instead of the methyl alcohol used with the acetone, one may use any lower aliphatic alcohol such as ethyl, isopropyl or butyl alcohols. In the solvent extraction under acid conditions, one may alternatively wash the estrogenic material with ethylene dichloride in place of the benzene and ether. Instead of using saturated sodium chloride solution, other alkali metal saturated salt solutions may be used provided these salts are relatively non-toxic. Preferred salt solutions are the sodium and potassium sulfates, phosphates and chlorides.

In the use of buffering agents to impart stability to the estrogenic product, one may use in place of the sodium citrate of the examples any non-toxic buffering agent which is soluble in blood fluids and capable of providing a pH within the range of 6.5 to 7.5, preferably on the slightly alkaline side. With regard to the carrier or drying agent, in place of lactose one may use any non-toxic material that is water-soluble, soluble in blood fluids and capable of giving body and helping in the drying of the product. Besides lactose, preferred carrier agents are those which have been mentioned earlier. With regard to the topical cream medicament, while a standard ointment base of the vanishing cream type was given in the example, one may also utilize a non-drying cold cream containing the standard and well-known ingredients for this type of product.

WHAT WE CLAIM IS:—

1. The process for obtaining an estrogenic product of high potency and substantially free of toxic impurities from pregnant mares' urine wherein a substantial amount of the estrogenic content is extracted from the pregnant mares'

urine by use of selective extracting agents thus obtaining an estrogenic concentrate, characterized by dissolving the estrogenic concentrate in an aqueous medium and adding an inorganic salt in an amount sufficient to precipitate the estrogenic fractions substantially free of toxic impurities orginally present in the concentrate, removing said precipitate, and selectively separating the desired estrogenic components from inorganic salt in said precitate.

2. The process of Claim 1; wherein the inorganic salt used as the precipitating agent is an alkali metal salt of either sulfuric, phosphoric or hydrochloric acid.

3. The process of Claims 1 or 2; wherein inorganic salt in the precipitate is selectively separated from the estrogenic components by use of an organic solvent comprising an aliphatic ketone containing a small amount of aliphatic alcohol which selectively extracts the estrogens.

4. The process of Claim 3; wherein the organic solvent is separated from the estrogenic components by adding water to the solvent extract and distilling the mixture to remove the organic solvent.

5. The process for obtaining an estrogenic product as particularly described with reference to Example 1.

6. The process for obtaining an estrogenic product as particularly described with reference to Example 2.

7. The process for obtaining from pregnant mares' urine, an estrogenic product of high potency and substantially free from toxic impurities substantially as described hereinbefore.

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